Guidance on Cholinesterase Measures in DNT and Related Studies (10/29/01)

The purpose of this paper is to provide OPP's views on the measurement and use of cholinesterase (ChE) inhibition data to:

- characterize the adequacy of postnatal dosing in developmental neurotoxicity studies, and;
- define the comparative sensitivity of adults and young organisms to support risk assessments under FIFRA and FQPA.

Our goal is to facilitate discussion with the American Crop Protection Association (ACPA) and other interested parties on the conduct of these studies to provide data to satisfy the Data Call-In (DCI) for adult and developmental neurotoxicity (DNT) studies for the organophosphorus pesticides with tolerances.

This document also includes, as background material: discussion of available data regarding fetal and pup exposure during DNT studies (Appendix 1); generic sections of the Agency reviews of registrant DNT protocols that relate to the measurement of ChE activity (Appendix 2); and an excerpt on ChE inhibition data from the working paper of 2/10/00 produced for the discussions that were held with ACPA in January-March, 2000 (Appendix 3). Their contents are still applicable to these studies, except that the working paper recommendations about the time of ChE measurements in the pups have been superceded in the generic or current reviews.

Assessing the adequacy of postnatal dosing

A major uncertainty in the evaluation of DNT studies is the extent to which continuing to dose the dams during lactation provides exposure to the offspring during this dynamic phase of neurological development. Under the September 10, 1999 DCI, registrants are required to assess the adequacy of post-natal dosing during the developmental neurotoxicity study. The following discussion addresses issues regarding determination of the adequacy of post-natal dosing.

In a developmental neurotoxicity study, pup exposure to test substance during the lactation period may occur via three pathways: 1) maternal transfer via milk; 2) consumption of treated diet by pups; and/or 3) direct dosing of pups. In studies where the dosage to the dam is by gavage, and during dietary studies prior to the onset of diet consumption by pups, postnatal exposure to pups depends exclusively on exposure through the milk. Available data indicate that exposure via milk is variable, both among compounds and across time for a given compound (some available data are discussed in Appendix 1). In dietary studies, postnatal exposure to pups may include some ingestion of test substance by pups during late lactation (available data indicate this ingestion may be very limited; see Appendix 1). The changing nature of pup exposure during this critical period means that adequacy of dosing for pups must be assessed repeatedly during the pre-weaning period.

Since available data indicate that exposure during lactation may vary over time, measurement of milk content of test substance, measurement of a biomarker (such as ChE inhibition), or presence of clinical signs (such as decreased body weight) at a single time point during lactation may not be sufficient to document adequacy of exposure throughout the lactation period. Further, the levels of

enzymes that may activate (e.g., Basu et al., 1971; Atterberry et al., 1997) and metabolize the organophosphorus pesticides change during the lactation period (see Figure 1, which documents developmental changes in levels of carboxylesterases and chlorpyrifos-oxonase) (Moser et al., 1998).

In the reviews of the draft protocols, EPA asked registrants to conduct preliminary studies to evaluate the adequacy of postnatal dosing (e.g., milk concentrations of the test substance, along with milk consumption and food consumption data in pups) as a means to determine the need for direct dosing. Alternatively, studies may seek to define the adequacy of postnatal dosing by evaluation of the available toxicity data in the pups during the main DNT study, e.g., survival, body weight, clinical signs, and ChE inhibition. Preliminary studies including these endpoints may play a critical role in assuring the adequacy of dosing for the main DNT study. In the absence of signs of toxicity or ChE inhibition in the pups during one or more critical phases of neurological development, the adequacy of the developmental neurotoxicity study may be called into question.

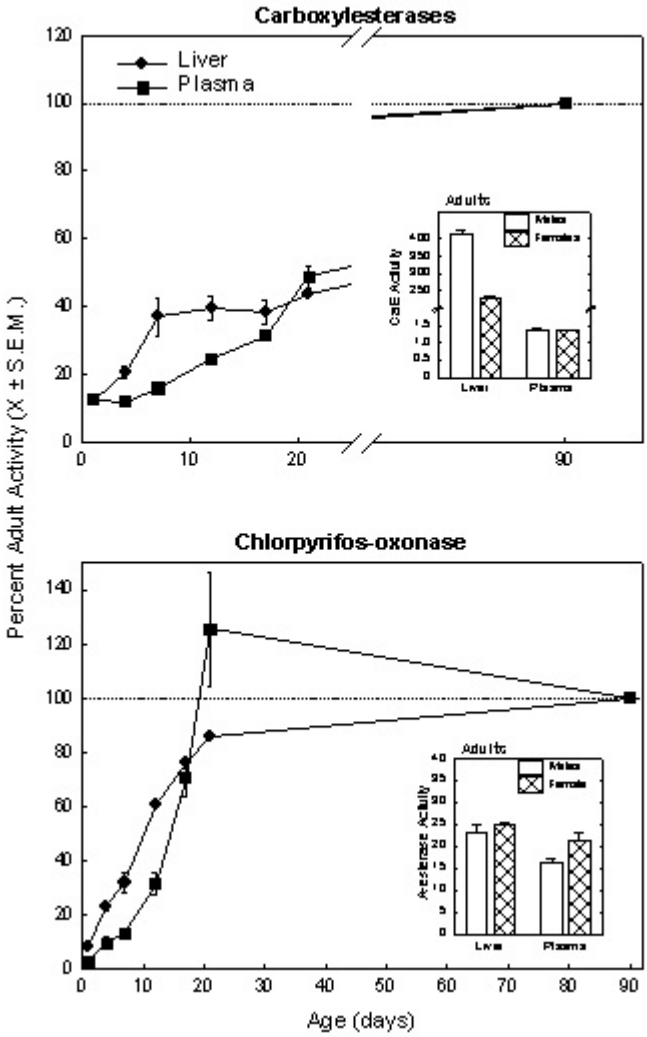


Figure 1. Ontogeny of carboxylesterase and chlorpyrifos-oxonase in rats.

Given that ChE inhibition is often the most sensitive endpoint for risk assessments of antiChE pesticides based on adult exposures, it should be an essential component in defining the potential developmental neurotoxicity of antiChE pesticides. Based on the variable nature of exposure during lactation (as discussed above), ChE inhibition should be assessed repeatedly, as is done for other measures, to follow changes in exposure and the effect seen throughout the three week lactation period. Thus, ChE inhibition plays an important role in defining the adequacy and pattern of exposure in the DNT study.

The pup ChE data gathered in the DNT study design, without data regarding dose to the pup, will not provide definitive data regarding comparative sensitivity, since the comparisons are relative and not

directly quantitative. These data do, however, provide important information on the pattern of exposure and/or effects in defining the adequacy of postnatal exposure and aid in interpreting other toxic effects.

Studies of Comparative Sensitivity

Comparative sensitivity data in pups and adults may provide reliable data (1) for determination of an alternative to a 10-fold margin of safety factor, applied under FQPA to protect infants and children, and/or (2) for use in selection of endpoints for specific risk assessments, i.e., acute or repeated exposures. ChE and exposure data collected in the DNT study, when sufficient to allow estimates of dose to the pups, could provide estimates of comparative sensitivity for use in risk assessment. Alternatively, such data may be acquired in separate studies.

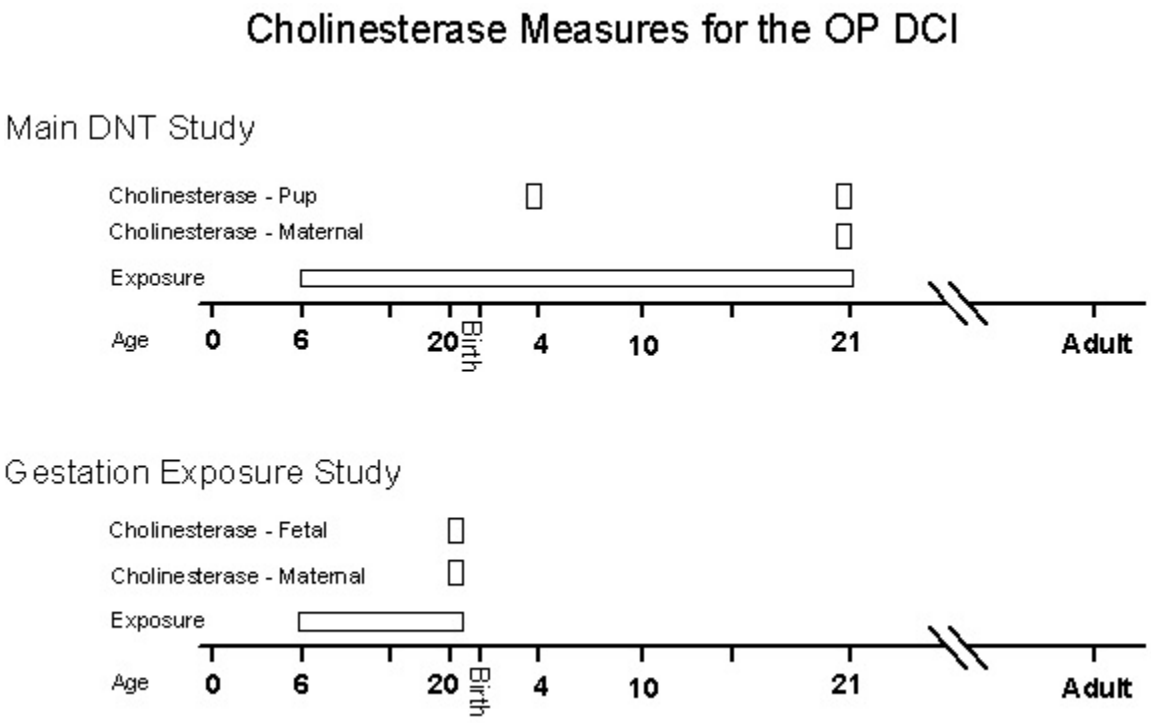
ChE activity should be measured in blood (RBC and plasma) and brain; measurements of peripheral nervous system tissues are also recommended. Assessments should be done at appropriate sampling times, with respect to test substance administration:

1) Peak effect time for acute studies (using preliminary studies to define time of peak effect);
2) Similar time point after administration for repeated dose studies;
3) Time points for gestational and lactational studies should allow for test substance distribution to fetal or milk compartments (and for transfer to pups during lactation).

For any OP the time of peak effect may vary as a function of age, since, as noted earlier, pups differ in the rate by which they synthesize ChE, and the level of activity of the enzymes that may activate and metabolize the OP under study. Clinical signs may predict the time of peak effect for an OP, and have been used in adult studies for that purpose. But clinical signs in pups would be more difficult to detect, have not been collected in the past by most labs, and only indirectly reflect the action of the OP. That is, their time course may reflect the influence of effects and variables not directly related to the extent of ChEI. It is also expected, based on the adult data, that OPs will vary in terms of the relation between the pattern of clinical signs seen and the pattern of ChEI. Thus, more comparable data between OPs will be provided based on ChEI. Since the primary focus of these studies is the ChE measures, it is preferable to define the time of peak effect for ChE measures.

For each type of study, the rationale and data supporting chosen sampling times must be provided. In repeated exposure comparative sensitivity studies, in addition to ChE measurements, it is recommended that traditional toxicity measures, such as survival, body weight, and age-appropriate clinical signs, be included.

Figure 2. Cholinesterase measurements in the main DNT study and the gestation only study.



Populations and Exposure Scenarios for ChE Assessments

In summary, based on review of the populations and exposure durations needed to support various aspects of dietary and residential risk assessments, and consistent with the terms of the DCI, there are a number of populations and exposures for which EPA seeks data.
Evaluations for these population groups could be performed in a separate study or as satellite groups to the main DNT study (study design is summarized in Figure 2).

1. Exposures to Pregnant and Lactating Women:

To provide data for use in risk assessment scenarios that include pregnant and lactating women, EPA recommends assessment of dams at the end of gestation (GD20) and at the end of lactation (PND 21). Both are important sub-populations of adults whose comparative sensitivity in relation to other adults or the young may be unique. GD20 measures in dams are relevant to the adequacy of dosing in the prenatal phase, and measures in lactating dams (e.g., PND21) are relevant to the adequacy of postnatal dosing to the dam, consistent with the acknowledged essential relevance of ChE measures in the assessment of the adequacy of dosing in any study of an OP.

Second, GD20 measures in dams are relevant to determining the relative sensitivity of the dam and the fetus, although this is a compromise in that the ratio of ChEI in the dam and the fetus is only an indirect measure of exposure (unlike concentrations of test material); the more rapid synthesis of ChE in the fetus may also lead to underestimates of relative peak levels of inhibition.

2. Exposure to Fetuses and Nursing Infants:

A. Fetal Measures on GD20

In addition to the comparison with the dams just discussed, fetal ChE effects can support judgments of the adequacy of prenatal dosing (in addition to effects in the dam). Measures of ChEI in the fetuses on GD20 also provide a baseline for comparison to lactational exposure measures in pups, as illustrated in the chlorpyrifos example (see Appendix 1). This comparison of the dam to the fetus on gestation day 20 defines a baseline relationship in effect when both exposures are *via* the blood. It is somewhat analogous to the use of the i.v. dose in metabolism studies. Postnatally, pup exposure is via the milk for the first weeks of life, a more complex and indirect exposure route. Using the GD20 fetal ChE data as a baseline, comparisons between the fetus and the pup, particularly on PND 4 can be made. In the absence of precise chemical concentration and thus dose data in the pups, we can only compare the postnatal ChEI data to the GD20 data. PND 4 measures may reflect some carryover of prenatal exposure as well as exposure via the milk. These relative comparisons help to describe the pattern of exposure, which is then evaluated as a whole, i.e., by the weight of the evidence for exposure during the pre- and post-natal periods.

Comparisons of fetal ChE between OPs may also be useful. While this comparison may not drive the reference dose, it still may be relevant to comparing the risks of two chemicals, e.g., if one affects fetal ChEI while another does not.

B. Nursing Infants

As reviewed above, it is essential to measure ChE inhibition in young rats throughout lactation. For the purposes of assessing adequacy of dosing in pups, as a practical compromise, EPA now will accept two measures of ChE in pups in the DNT study, during early and late lactation. In many of the designs presented in the draft protocols submitted by registrants, ChE will be evaluated in pups on PND 4, and in pups and dams on PND 21. EPA previously recommended (in its protocol reviews) that ChE measurements should be made in pups on at least one other time point during the lactation period. Based on many of the study designs submitted, EPA believes that sufficient pups are available for this measurement in the DNT study without the need for additional dams and would provide an important indication of mid lactation effects, likely less influenced by prenatal exposures than PND4 and prior to the onset of dietary exposure of the pups.

3. Direct Acute and Repeated Exposures to Young (Non-nursing infants and children 1-6):

A. Adequacy of Dosing for Comparative Sensitivity Studies

The primary goal of the comparative sensitivity studies is to determine both the NOAEL/LOAEL and some measure of the range of the dose effect curve, e.g. an ED50,
for all three compartments (plasma, red blood cells, and brain) in both young and adult animals. It is standard practice in toxicology studies of all kinds to use both sexes, and that is the recommendation here.

Identifying which sub-group and compartment has the lowest NOAEL/LOAEL may be essential for the selection of endpoints for the risk assessment. Determination of a reliable descriptor of each dose effect curve is essential for making comparisons between ages, which is essential for supporting an FQPA factor. Trying to compare NOAELs may underestimate or overestimate comparative sensitivity. What is needed is sufficient information from each dose effect curve to compare doses that cause a particular effect, e.g an ED20 in each compartment and age group. It is acknowledged that we are more concerned with defining the low end of the dose effect curve, e.g. the ED20 than the ED80.

Our current cholinesterase policy relies on a weight of the evidence evaluation of the data on all three compartments, i.e., plasma, red blood cells, and brain, and may rely on any of these under some circumstances to derive the critical effect. The pattern of effects between compartments affects the weight of the evidence, so it is important to know what that pattern is. The dose effect curves (and their descriptors) are the principal means to evaluate this pattern, and this in turn requires data from doses spanning some range of effects. This then, requires that we define the dose effect curve for each of the compartments to support our comparative sensitivity judgments.

The high dose for a given study should be chosen to cause sufficient inhibition to serve as the high end of the least sensitive of the 3 major compartments for that sex/age. It is recognized that this may not always be possible, and it should also be recognized that for some chemicals the slope of the dose effect curves for the different compartments may vary widely, e.g., malathion, and that more than 3 dose groups may be needed to adequately describe the dose effect curves for all three compartments.

There are a variety of ways that one may conduct measurements in the different age animals of both sexes, and a number of suggestions for more limited testing sequences have been made. Adequate adult data may exist from earlier studies. These data may show, among other things, a clear sex difference, or the lack of one, or that one compartment is more sensitive than the others. Pilot studies in pups may also be conducted and their data available. However, pilot data are not sufficiently reliable to obviate the need for more complete testing (for example, they usually lack sufficient numbers for statistical analyses). Similarly, data on the relation between sexes or compartments at one age may not predict that relationship at other ages. The ultimate goal is to describe the comparative sensitivity for both sexes of animals at different ages (e.g., to compare the sensitivity of adult females and day 4 females, as well as adult males and day 4 males, for each compartment). More limited testing strategies, such as data from one compartment, or data from one sex, cannot reliably be defined, because there are so many patterns of results that may occur, and because it is not feasible to anticipate them all .

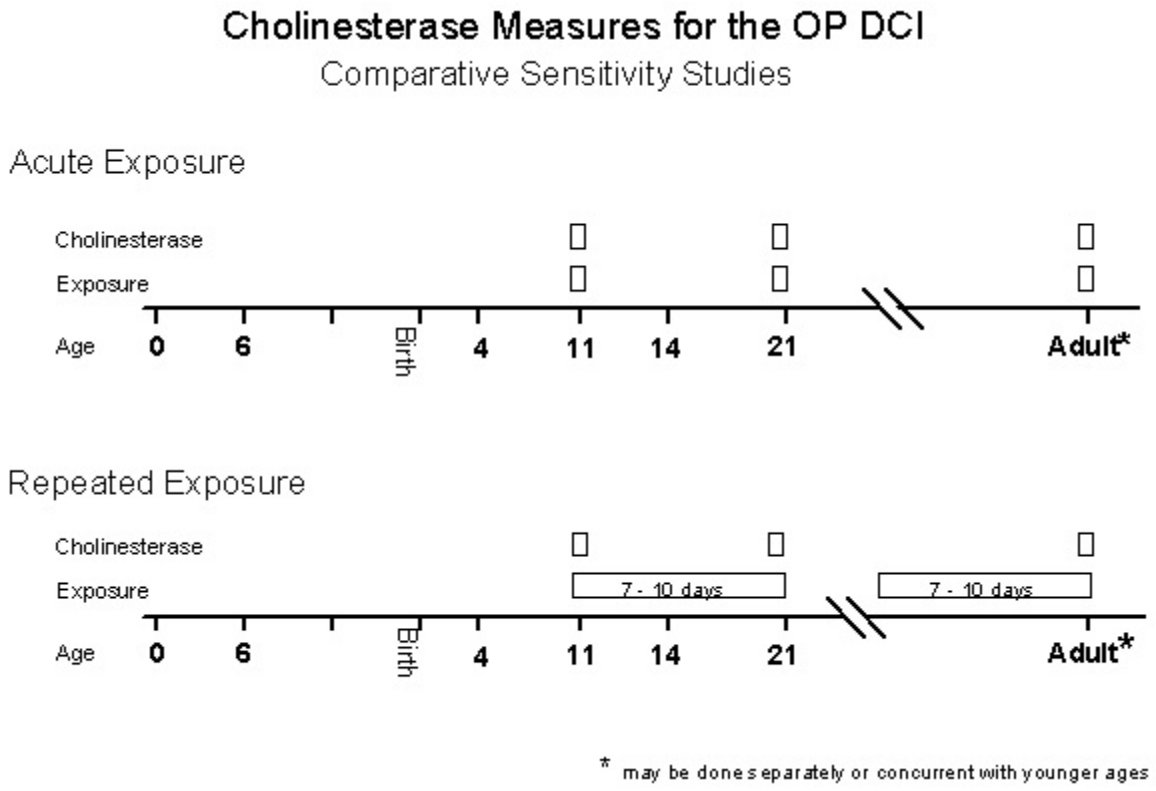


Figure 3. Cholinesterase measurements in the comparative sensitivity studies.

B. Timepoints for Directly Dosed Pups

A straightforward approach to assess age dependent differences in sensitivity is a study using acute and short term oral exposures to animals of different ages. Data collected from such experiments will allow for direct comparisons of toxicity of the test substance at different ages. There are numerous examples of such studies (Benke and Murphy, 1975; Stamper et al., 1988; Pope et al., 1991; Zheng et al., 2000; Beyrouty et al., 2001).

EPA recommends that the following populations be evaluated in the separate comparative cholinesterase study (the study design is also presented schematically in Figure 3):

1. Acute (single dose) studies:

A. Rationale.

Available data indicate that, in some cases, pups are more sensitive than adults to cholinesterase inhibition caused by organophosphate pesticides (e.g., Atterberry et al., 1997; Benke and Murphy, 1975; Beyrouty et al., 2001; Moser et al., 1998; Pope et al., 1991). While limited (representing perhaps 5 out of 20+ registered organophosphate pesticides), these data indicate that when increased sensitivity is

present, the magnitude of difference is greater at earlier time points than at later ones. Registered organophosphate pesticides vary widely in their structure and activity: different compounds are metabolized by different groups of enzymes; some require metabolism to the active form, others are active as applied. Adult levels of metabolizing enzymes are not present in rat pups at birth; the age at which adult levels are reached, as well as the time course of development, varies among enzymes and across compartments (e.g., liver, plasma). EPA will accept evaluation of comparative sensitivity at two time points during lactation and one adult time point: the first lactation time point should be no later than PND11, the second lactation time point should be 7-10 days later (corresponding to the final day measured in the repeated dose study). Evaluation of data for these two time points will provide specific information regarding differences in sensitivity between pups and adults for two different ages of pups, as well as some information about the direction and magnitude of the change over time.

B. Specific Design.

1. Pre-weaning pups (both sexes);
   - Early-Mid lactation [no later than PND11];
   - Late lactation [7-10 days after first time point, no later than PND 21];
2. Young adults (both sexes).

2. Repeated dose studies:

A. Rationale.

It has been demonstrated for several compounds that levels of cholinesterase activity return to control levels more rapidly in pups than in adults following exposure to organophosphate pesticides (cf Pope et al., 1991). This rapid return to control levels is probably due to more rapid synthesis of cholinesterase during development, rather than to differences in duration of inhibition of individual enzyme molecules. Because of this more rapid synthesis of cholinesterase in pups, it has been hypothesized that pups will be less sensitive than adults to repeated exposure to organophosphates (i.e., less cumulative inhibition will occur, due to more rapid 'recovery' between exposures). Although this hypothesis appears to be valid for some chemicals, greater inhibition in pups than in adults has also been seen following repeated OP exposure (cf Beyrouty et al., 2001). Since the presence or absence of increased sensitivity following repeated exposure is known to vary across chemicals, data regarding comparative sensitivity following acute exposure will not be sufficient to allow prediction of relative sensitivity following repeated exposure. Therefore, comparative sensitivity studies should include repeated as well as acute exposures. The time point (i.e., post-natal day) assessed at the end of the repeated exposure should correspond to one of the time points assessed following acute exposure (see above). Comparison of the results from these two studies will enable specific determination of whether repeated exposure leads to cumulative cholinesterase inhibition in pups.

B. Specific Design.

1. Pre-weaning pups -- exposure beginning during early lactation, with a duration of 7-10 days (starting no later than PND 11, e.g. PND 11-21), with ChE evaluations at time of peak effect after dosing on last day of exposure;

2. Young adults (both sexes) -- repeated dose exposure using duration and doses as for pre-weaning pups, with sampling at time of peak effect.

References

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APPENDIX 1

The most reliable means to define postnatal dosing is to directly dose the pup for some or all of early life. In a series of studies Chapin et al. directly dosed pups beginning on postnatal day 7 (Chapin et al., 1997; Moser et al., 2001). Studies conducted by Cheminova, following EPA's recommendation, on methyl parathion, malathion, and dimethoate, directly dosed pups by gavage from postnatal day 11 through 21 (e.g., Beyrouty et al., 2001). HED scientists have also visited the laboratory [CTBR] of Beyrouty et al. who demonstrated their procedure for gavaging pre-weanling pups. The procedure appeared to be well established, and was described by the CTBR staff as effective in pups as young as four days old.

A number of factors affect exposure through the milk, which may vary for each test substance (Dorman et al., 2001). Transfer of a chemical across the blood milk barrier, is affected by molecular size, lipophilicity, and pH; the rate of transfer across the mammary epithelium and the plasma half life. Pup exposure is also affected by the rate of gastrointestinal absorption as well as distribution, metabolism, and excretion. Additionally, the test chemical may alter milk secretion and composition.

The concentration of test substance in the milk is known to vary within the 3 week lactation period. In a study of chlorpyrifos (Mattson et al., 1998) the concentration of test substance in the milk decreased across the first 5 days of life. Measures of chlorpyrifos in milk were made in 5 dams/dose on GD 20, and LD 1, 5, and 11(one day after the last dose) and are shown in Table 1. Milk concentrations decreased by about 50% between day 1 and day 5, and almost completely disappeared on day 11, one day after dosing stopped.

TABLE 1. Milk concentrations of Chlorpyrifos (ng/g) in pups during lactation.

DOSE (mg/kg/day)	**0.3**	**1**	**5**
PND 1	20.57	139.49	3022
PND 5	13.54	81.76	1533.98
PND 11 (after dosing)	ND	ND	19.79

ND = non detect.

Brain ChE inhibition from high dose pups showed a similar pattern, as shown in Table 2.
(No effects on Brain ChE activity were seen in pups from the low- or mid-dose dams.)
The ChE data show considerable recovery in comparison to the levels found at the end of gestation, and continue to recover through the first five days of lactation, while exposure to the dams continued. This indicates that exposure to the test substance in the milk likely decreased early in lactation relative to gestation and during lactation days 1-5. If measures had been made only on day 5, this pattern would not be apparent.

TABLE 2. ChE data (% inhibition) from high dose pups.

	Hindbrain	Heart	Plasma
GD 20	53.9%	81.6%	84.7%
Day 1	32.8%	65.3%	60.0%
Day 5	11.6%	16.1%	19.5%

In dietary studies, postnatal exposure to pups may also entail some ingestion of test substance, in treated diet, by the pups during the last week of lactation. One study that measured the ontogeny of food consumption in the Sprague Dawley rat showed onset of eating on PND 18 with a gradual rise over the succeeding 5 days. On day 21, intake was 3 g/pup (Gerrish et al., 1998). A second study in Fischer 344 rats also found onset of eating on PND 18 (0.6 g/litter[8 pups/litter]), and 17.9 g on day 21 [2.2 g/pup], 45 g on day 24, and 60.7 g on day 27 (Hanley & Watanabe, 1985). The results of these studies indicate that direct dietary exposure to pups may be minimal prior to day 18 of lactation, and increases rapidly after that point.

Some additional data demonstrating patterns of cholinesterase inhibition in pups is summarized in Table 3.

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Note: The examples in Table 3 are presented for illustrative purposes only. While the examples are based in part on real findings, the data used in these examples are preliminary; the examples do not represent final Agency review or evaluation of any submitted data.

Table 3. Examples of "adequacy of dosing" issues raised by results of DNT studies conducted with varying protocols and test compounds.

	ChE data available from main study		ChE results from main study		Other effects from main study		
Exposure Scenario	Dams	Pups	Dams	Pups	Dams	Pups	Comments
Dietary to dams only, GD6-PND21	PND21	PND11 PND21	↓↓plasma, rbc, brain at mid and high dose	slight ↓brain PND11 high dose ♀; slight ↓plasma, PND21, high dose ♂♀	clinical signs; slight ↓body weight during lactation at high dose only	↓body weight PND4-end; ↓aud. startle, ↓brain weight and measures [high dose only]	Dosing may be adequate based on decreased pup body weight at high dose starting PND4; Relative dose to pups unknown, but likely much lower than dams (based on relative ChE inhibition); Possible increased sensitivity of pups, based on increase in other types of toxicity at low levels of ChE inhibition.
Gavage to dams, GD6-PND11, gavage to pups PND11-21	GD20 [also adults following single or 11-day dosing]	GD20, PND4, 11, 21	↓plasma at high dose, ↓ rbc, brain at mid and high dose	GD20-no change [slight ↓ plasma high dose females, slight ↓rbc high dose both sexes]; PND4-no change; PND11,21-↓↓plasma, rbc, brain [mid & high dose]	Not available	Not available	No gestational ChE inhibition; early lactational exposure not demonstrated (probably low, based on lack of ChE inhibition); increased sensitivity to direct dosing on PNDs 11 and 21, based on ChE inhibition.
Gavage to dams, GD6-PND11, gavage to pups PND11-21	GD20 [also adults following single or 11-day dosing]	GD20, PND4, 11, 21	↓plasma, rbc, brain at high dose; ↓ brain at mid dose	GD20: ↓brain at all doses; ↓plasma, rbc at high dose; PND4:less ↓than GD20; PND11,21: ↓brain at all doses day 21, mid dose and high dose day 11; ↓plasma, rbc at high dose	none	possible slight ↓pup wgt at high dose; ↑pup death at day 4, mid and high doses; possible ↓ activity, delayed righting response in high dose	Dosing adequate based on pup ChE inhibition, mortality, and possible other effects. Increased sensitivity of pups, based on mortality and brain ChE inhibition.

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	ChE data available from main study		ChE results from main study		Other effects from main study		
Exposure Scenario	Dams	Pups	Dams	Pups	Dams	Pups	Comments
Gavage to dams, GD6-PND11, gavage to pups PND11-21	GD20, PND4 [also adults following single or 11-day dosing]	GD20, PND4, 11, 21	GD20: ↓ rbc at mid and high dose; PND4: no effects	GD20: ↓ rbc at mid and high doses; slight ↓ plasma at mid and high dose; no change in brain; PND4: no inhibition; PND11, 21: ↓rbc, plasma at mid and high doses (slight ↓ rbc at low dose); ↓ brain at mid-high and high dose	not available	not available	Dosing adequate during gestation and late lactation based on ChE inhibition; not demonstrated during early lactation in dams or pups. Increased sensitivity of pups based on ChE inhibition.

APPENDIX 2

Generic guidance on ChE measures from previous draft protocol reviews.

It is recommended that the final protocols contain chemical-specific information to justify the timing of sample collection, e.g., the time of peak behavioral effect in the adult acute neurotoxicity study.

Pooling samples:

Pooling samples from pups should not be necessary. Pooling fetal samples, in order to attain a sufficient volume of tissue for cholinesterase measurement, may not be necessary. However, if pooling fetal blood samples is necessary, combine only samples from fetuses within the same litter, and not from different litters.

Peripheral nervous system (PNS) cholinesterase measurements:

Measurements of cholinesterase inhibition in a variety of PNS tissues are highly recommended, following the FIFRA Scientific Advisory Panel (SAP) advice supporting the feasibility of measuring ChE inhibition in PNS tissues (US EPA, 1997), and the International Life Sciences Institute (ILSI) project (Mileson et al., 1999) which provided further guidance on these measures. The ILSI guidance document discusses several tissues of potential utility, including the atria of the heart, skeletal muscle, lung, diaphragm, and salivary glands. With their potential use as alternatives to blood measures, measurements of ChE inhibition in PNS tissues provide an opportunity for a broader consideration of blood measures *vis a vis* PNS targets for risk assessment.

Methodology for cholinesterase assays:

Discussion of specific methodology for the assay is contained in the Proceedings of the U.S. EPA Workshop on Cholinesterase Methodologies, dated March 1, 1992, and should be consulted for guidance on those issues in preparation of the protocol. Also, see more recent papers by Wilson et al. (1996) on organophosphates and Hunter et al. (1997) on carbamates.

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APPENDIX 3

Excerpt from A Working Document: OPP Comments on the DNT DCI for OPs Dated 2/10/00

3) Cholinesterase inhibition data

A requirement for comparative evaluation of cholinesterase inhibition in dams and offspring was included in the DCI for OP Developmental Neurotoxicity Studies. It states: "Measurements of brain, red blood cell, and plasma cholinesterases should be made in both dams and pups in a sufficient number of animals and with sufficient frequency to characterize the comparative levels of inhibition, and both the time of peak effect and recovery."

The following is meant as initial feedback on OPP's thinking on this requirement.

- Based on well-conducted studies, 5 dams/dose and 5 pups/sex/dose at each time point should generally be sufficient. In our experience (Ellman method), statistically significant changes on the order of 5-10% in brain,10% in red blood cells, and 20% in plasma should be detectable. We have seen studies submitted with far greater changes failing to achieve statistical significance. If data are more variable than would allow the detection of changes in those ranges, larger group sizes and/or a review of the assay methodology should be undertaken.

- The DCI calls for measurements in plasma, red blood cells, and brain cholinesterase, consistent with current policy on their use for risk assessment. However, PNS tissues (e.g., heart, diaphragm) are highly recommended, following the SAP advice supporting the feasibility of PNS tissues, and the ILSI project which provided further guidance on these measures. With their potential use as alternatives to blood measures, measurement of PNS tissues would provide an opportunity for a broader consideration of blood measures *vis a vis* PNS targets for risk assessment.

- "Time course and recovery" refers to when to take the samples with respect to dosing at the time of peak effect following an acute dose or series of doses. These measures must balance between the desire to make these measures as comparable as possible between dams and pups, i.e., at the same time after dosing, while recognizing that the time of peak effect may vary between dams and pups, and for pups as they mature. It may be preferable to assess a small number of animals of different ages in a structured way for estimation of the time of peak effect prior to the main study.

The risk assessment will need to address the potential risk of developmental prenatal exposures, lactational exposures via milk, and/or mixed with early dietary exposure, and both acute and subacute oral exposures to developing organisms in comparison to adults. Based on general existing knowledge of the ontogeny of lactational exposures, food consumption, and metabolizing enzymes, we would suggest measures at the following times as representative of these stages:

- Overall Gestational Exposure on GD21.

- Early Lactational Exposure on PND 1, 5, and 11;
- Late Lactational and Early Eating Period on PND 17, and 21.

During gestation and lactation, comparisons are to the dam. But from postnatal day 11-21, this is more complicated. Pups in dietary studies may have mixed milk and dietary exposures. Data from directly dosed pups could be compared to data from adults that were dosed directly.

Pharmacokinetic measures, such as milk levels, or food consumption, or biomarkers such as liver carboxylesterases (Lassiter et al., 1999) should be coordinated with these time points.